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(54) Title: PRODUCTION OF VANILLIN

(57) Abstract

A method of producing natural vanillin comprises reacting vanilla plant root material with ferulic acid or derivatives thereof (preferably ferulic acid derived from condensed molasses solubles) in the presence of adsorbent material (preferably charcoal) that releasably adsorbs vanillin but does not adsorb ferulic acid or derivatives thereof; and extracting vanillin from the adsorbent material, e.g. by solvent extraction. Ferulic acid is a precursor of vanillin, and the vanilla plant root material acts as a biocatalyst catalysing production of vanillin from ferulic acid (or derivatives thereof). The adsorbent material acts as a product reservoir for the vanillin produced, thus relieving possible product inhibition and/or further metabolism. The reaction also produces p-hydroxybenzaldehyde, at least some of which can be releasably adsorbed on the adsorbent material. The invention also provides a novel product comprising vanillin and p-hydroxybenzaldehyde at a vanillin: p-hydroxybenzaldehyde weight ratio of about 7.8:1.

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Production of Vanillin

Field of invention

This invention concerns the production of vanillin and relates to a method of producing natural vanillin suitable for use as vanilla flavour and the vanillin so produced.

Background to the invention

Vanilla/vanillin is very widely used as a flavour, for instance in ice creams, a very wide range of foods, and also in some fragrances. Vanilla beans contain about 2-3% (w/w) vanillin (reference 1), and typically cost about US$70/lb. Vanilla bean extracts contain vanillin as a major component but also contain a variety of other related materials including p-hydroxy benzaldehyde (pOHB), 4-oxy-benzyl alcohol, vanillyl alcohol, coumaric acid, 3,4-dihydroxy-benzaldehyde, vanillin acid etc. (reference 5), many of which contribute to the high organoleptic quality of the extracts. Vanilla bean extracts currently vary in price from about US$ 100-300/Kg depending on quality, especially vanillin content. The markets for these natural products and for nature-identical synthetic material are about US$100M and US$80M per annum, respectively, so that although the natural vanilla market is only 13% of the total by weight, it represents some 56% of the market by value. This higher price is a reflection of the much higher organoleptic value of bean-derived vanilla flavours. Thus the vanillin present in vanilla beans can be calculated to have a value of about US$2,500/Kg.

Vanilla is a vine orchid that is very difficult to grow as a commercial crop because of its exacting growth requirements, and also because of the need to hand-pollinate the flowers which are only fertile for one day. Hence there is a significant need for alternative commercial processes for producing natural vanilla or vanillin, that are not subject to variations in supply,

quality and price.

Several approaches to the production of natural vanillin have been pursued. BASF have submitted a patent application for the biotransformation of eugenol into vanillin using an Arthrobacter globiformis strain that does not further metabolise the vanillin via vanillic acid (reference 2). Similarly Serratia and Klebsiella strains have been used acting on eugenol and isoeugenol (reference 6) and Paecilomyces variotti can produce vanillin from ferulic acid (reference 7).

Two approaches using plant cell technology have been reported: callus cultures (reference 8) and vanilla cells selected for high production and secretion capability (reference 4). The pathway of vanillin synthesis, however, is not fully characterised yet (reference 3). Preliminary work showed that when phenylalanine was used as a precursor considerable amounts of p-coumaric acid accumulated, indicating that its metabolism was a rate limiting step in the pathway and ferulic acid was the best precursor for further metabolism to vanillin.

Summary of the invention

In one aspect, the present invention provides a method of producing natural vanillin, comprising reacting vanilla plant root material with ferulic acid or derivatives thereof in the presence of adsorbent material that releasably adsorbs vanillin and extracting vanillin from the adsorbent material.

Ferulic acid (3-methoxy-4-hydroxy-cinnamic acid) is a precursor of vanillin (4-hydroxy-3-methoxy-benzaldehyde), and the vanilla plant root material acts as a biocatalyst catalysing production of vanillin from ferulic acid (or derivatives thereof). The adsorbent material acts as a product reservoir for the vanillin produced, thus relieving

possible product inhibition and/or further metabolism. The reaction also produces pOHB, at least some of which can be releasably adsorbed on to the adsorbent material.

The root material is conveniently in the form of small pieces, e.g. about 5mm lengths, although the form and size of the root material is not important. Indeed, it is possible to use whole plantlets. The root material may be obtained from plants grown conventionally, e.g. in a glasshouse, or produced by tissue culture.

The adsorbent material may comprise a suitable ion exchange resin or other material. However, the currently preferred adsorbent material is charcoal, which is readily and cheaply available and has suitable adsorbent properties.

The adsorbent material is preferably in finely divided or powdered form. The adsorbent material is desirably in close physical proximity to, and preferably in direct contact with, the root material. The adsorbent material is preferably present at the start of the reaction, rather than being added later, as this is found to give better yields of vanillin.

The root material, ferulic acid (or derivatives) and adsorbent material are preferably incubated together under suitable conditions, e.g. in suitable growth medium containing sucrose, in an illuminated growth room at about 25°C under suitably sterile conditions with gentle agitation, for a period of about 3 days. The adsorbent material is then removed and treated to extract the produced vanillin therefrom, while leaving unreacted ferulic acid (or derivatives) adsorbed thereto. The adsorbent material can be recycled for further use. The root material can then be subjected to further treatment with ferulic acid (or derivatives) for a further 3 days with a new batch of adsorbent material (or the original

batch after vanillin removal). The process can be repeated several times, although the activity of the root material gradually declines with reuse, and in practice it is found that after at most 6 three day treatments vanillin production has fallen to such a level that further treatment is not worthwhile. After the final 3 day treatment, vanillin is extracted from both the root material and the adsorbent material and the spent root material discarded. The vanillin extracted after the various treatments can be pooled.

Vanillin is conveniently extracted from the adsorbent material (and also the root material) by mild alkaline hydrolysis, e.g. by treatment with 0.2 M KOH, followed by selective solvent extraction, e.g. using diethyl ether. This extracts vanillin and some pOHB but not ferulic acid. Ether extraction was found to be very much more efficient than ethanol/water extraction, even when using specialist extraction equipment. The resulting extract may then be dried to produce a product rich in natural vanillin and suitable for use as natural vanilla flavour.

The mild alkaline hydrolysis treatment has the effect of liberating the vanillin in soluble form. This indicates that the vanillin produced by the method of the invention is substantially different to the vanillin glucoside formed in vanilla beans (reference 2) since virtually all the vanillin present as vanillin glucoside can be readily liberated by acid or beta-glucosidase treatment, whereas acid hydrolysis treatment liberated only 20% of the vanillin produced by the root material. The conditions used were not, of course, sufficiently severe to release vanillin from lignified tissues.

Any unreacted ferulic acid (or derivative) remains adsorbed to the adsorbent material and can be recycled for further use. This allows for potentially complete conversion of the

ferulic acid. High conversion is economically important because the ferulic acid is a significant cost component in the overall process and because complete conversion of ferulic acid in one reaction can only be achieved using very low concentrations of ferulic acid (less than 10ug/g dwt tissue) due to substrate and/or product initiation effects and due to the known toxicity of ferulic acid to plant tissues. Also the vanillin to pOHB ratio of the product produced at these very low ferulic acid concentrations is significantly lower.

Ferulic acid or derivatives such as esters, e.g. ethyl ferulate, may be used in the method of the invention. However, ferulic acid itself is the favoured material as it is most readily available.

Ferulic acid is desirably isolated from condensed molasses solubles (CMS), which represent an economically attractive source of natural ferulic acid. CMS contains about 125g ferulic acid/tonne. Ferulic acid is conveniently isolated from CMS by treating CMS with industrial grade charcoal in finally divided or powdered form, either batch-wise or using charcoal packed into columns. The charcoal and extracted ferulic acid can then be used directly in the method of the invention, with that charcoal constituting the absorbent material.

The treated CMS can then be recycled for use as an animal feed supplement, if desired, because the protein content and total dissolved solids are essentially unaffected by removal of ferulic acid, so the product specification of CMS relevant to its use as an animal feed supplement is substantially unchanged. In this case, the real cost of the ferulic acid is equivalent to the cost of isolating it from the CMS. Ferulic acid is also known to be present in very low concentrations in a number of beet molasses, but these represent an economically less attractive source of the

material.

The invention also includes within its scope vanillin produced by the method of the invention.

Using the method of the invention, vanillin productivities of about 400mg/Kg(dw) tissue/day and concentrations of about 7g Kg^{-1} of root tissue can be regularly obtained. This concentration is about 35-fold greater than the concentrations of vanillin originally present in the root tissue and is about 40% of that present in matured vanilla beans. Using roots supplied with ferulic acid, vanillin is produced 5-10 times faster than its normal synthesis in vanilla beans or in roots that have not been supplied with precursor. The composition of the vanilla flavour produced using the method of the invention is comparatively close to that of vanilla beans; in particular it contains p-hydroxy-benzaldehyde (the second most important component of vanilla flavour) at a vanillin: pOHB weight ratio of about 7.8:1, as compared to a ratio of 12.8:1 for bean derived vanilla. This may impart a superior organoleptic value and make the product of this root process more valuable.

In a further aspect, the invention also provides a product comprising vanillin and p-hydroxybenzaldehyde at a vanillin : p-hydroxybenzaldehyde weight ratio of about 7.8:1.

The invention will be further described, by way of illustration, in the following Example and by reference to the accompanying drawing in which:
Figure 1 is a schematic flow of a preferred method of the invention.

Example

Plant Material

Axenic tissue culture plantlets of Vanilla planifolia were maintained on hormone free Murashige and Skoog medium containing 2% sucrose and solidified with agar. Roots were harvested from such cultures aseptically, weighed and diced into approximately 5mm lengths. Some glasshouse grown roots were also used.

Reaction Mixture

25g of roots was added to 20g dry heat sterilised powdered charcoal (Darco G60 from Aldrich Chemicals) in Murashige and Skoog medium containing 2% sucrose and 500μ moles of ferulic acid which was filter sterilised into the autoclaved medium. The mixture was incubated in an illuminated growth room at 25°C on an orbital shaker set at 100 rpm.

Harvest Extraction and Analysis

The medium and charcoal were decanted away from the roots which were washed to remove as much charcoal as possible. The roots were recycled with fresh medium and charcoal or extracted directly depending on the experiment. Filtered charcoal or washed roots were extracted as follows: Added to alkaline methanol (1M KOH/methanol 1:4) at a ratio of 1g wet weight to 5mls and heated at 80°C for 10 minutes. The alkaline methanol was recovered from the tissue/charcoal by centrifugation and the methanol evaporated at 80°C under vacuum. After cooling the extract was acidified to pH5 or less with 10M HC1 before extraction with diethyl either (20% of volume twice). The ethereal layers were collected, pooled and evaporated. The resultant material was taken up into water and aliquots analyzed for vanillin and p-hydroxy-benzaldehyde against authentic standards using reverse phase HPLC in known manner. Results are means of at least three replicates.

Using these techniques, a number of experiments were carried out and the following results were obtained.

RESULTS

Initial analysis showed that roots contain the highest concentration of vanillin compared to other somatic tissues (200µg/g DW). The addition of ferulic acid increases the vanillin concentration to 489µg/g after a 72 hour incubation. No increases could be seen after only 24 hours. The ferulic acid concentration in the tissue increases twenty fold; however, the conversion rate is only 1.5% (Table 1).

80% of the vanillin produced from ferulic acid required extraction with KOH in order to liberate it in a soluble form: this is substantially different to the vanillin glucoside formed in vanilla bean (reference 2) since virtually all the vanillin present as vanillin glucoside can be readily liberated by acid or beta-glucosidase treatment, whereas acid treatment only liberated 20% of the vanillin produced by the aerial roots. Synthetic ethyl ferulate was also metabolised by the aerial roots.

Considerable increases in the rate of synthesis of vanillin and the concentrations of vanillin produced could be achieved by adding charcoal (Table 1).

The results of a number of experiments are summarised in Table 1. In the column headed V, for rows 3, 4 and 5 three figures are given: the top figure is root vanillin content, the middle figure is total vanillin content, and the bottom figure is charcoal vanillin content.

The addition of ferulic acid increased the vanillin content of the root tissue over a three day period. However, in the presence of charcoal, root vanillin content was reduced but overall vanillin production was higher when the charcoal

was extracted. Over a five day period with a change of charcoal after three days productivity was higher. The final charcoal sample which included that closely associated with the roots contained most vanillin.

In the presence of charcoal, vanillin and some other components as p-hydroxybenzaldehyde (pOHB) adsorb to the charcoal.

Charcoal has several beneficial effects. The inclusion of charcoal greatly increases the amounts of vanillin produced, and hence the volumetric productivity of the reactor, presumably by acting as a product "sink" and thereby relieving product inhibition and/or further metabolism. The amounts of vanillin formed are selectively increased as compared to other metabolites such as p-hydroxybenzaldehyde; therefore the vanilla flavour produced has a composition closer to bean vanillin flavour than that produced in the absence of charcoal. The addition of charcoal has little apparent stimulatory effect on p-hydroxybenzaldehyde production and most of the p-hydroxybenzaldehyde was found associated with the plant root tissue rather than the charcoal.

Some charcoal becomes closely physically associated with the root tissue and probably mediates precursor and product transfer into and out of the tissue, thus overcoming rate-limiting steps in the reaction. There is some evidence that much of the vanillin produced is present in this closely associated charcoal. Certainly yields of vanillin were significantly reduced when the charcoal was maintained physically separate from the root tissue, and when the charcoal was added during the reaction rather than being present from the start. In addition, even after 1 day of a 3-day reaction substantial amounts of vanillin were already present in the charcoal.

In addition, reuse of the root tissue is facilitated by the use of charcoal. Greater yields of vanillin were obtained upon reuse of the root tissue, perhaps due to adaptation of the tissue, or more probably because of carry-over of some of the closely associated charcoal that contains much of the vanillin produced. In these experiments some of the root tip material continued to grow, but this new plant material probably does not make a significant additional contribution to the ferulic acid transforming activity of the aerial roots.

The aerial root tissue was reused four times over a two week period. Rates of vanillin formation of 400mg.Kg^{-1} dw/day were obtained which is about 5-fold greater than occurs in vanilla beans; and a concentration of 7g vanillin Kg^{-1} dw tissue obtained which is about 40% of that present in vanilla beans. These rates and productivities are also far in excess of the corresponding values quoted for plant cell culture or plant callus tissue culture techniques (references 4 and 10). However, only low intracellular concentrations of ferulic acid could be detected indicating that its transfer into the root tissue is rate-limiting.

In addition although the original medium contained 2.0g/l sucrose, no reduction in vanillin production occurred when this sucrose was reduced to 0.1g/l so as to reduce media costs.

The size of the root pieces used had little effect on activity; indeed, initial experiments show that whole plantlets can be used for the conversion of ferulic acid into vanillin.

The material produced by the roots in the presence of charcoal contained concentrations of p-hydroxybenzaldehyde in a ratio to the vanillin present that is quite close (7.8 vanillin: 1 pOHB) to that present in vanilla bean extracts

(12.8:1). The other minor components of vanilla such as vanillic acid are not present. Some residual ferulic acid remains, but this can be removed by treatment with suitable resins such as Amberlite A26 or 27.

Most of the vanillin formed and some of the p-hydroxy-benzaldehyde partitions into the charcoal from which it can be easily extracted in a purer and more concentrated form. Extraction with diethyl either is preferred because it selectively extracts the vanillin, but not the remaining ferulic acid. Thus the charcoal and remaining ferulic acid can be recycled and reacted. This feature of the process allows potentially complete conversion of the ferulic acid.

As mentioned above, the ferulic acid is desirably derived from condensed molasses solubles (CMS), which are available commercially, e.g. from Distillers. A preferred production route for producing vanillin by the method of the invention, based on extraction of ferulic acid from CMS, is illustrated schematically in Figure 1.

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TABLE 1

Total vanillin (V), ferulic acid (FA) and p-hydroxy-benzaldehyde (p.OHB) content of vanilla tissues and charcoal after alkaline hydrolysis (µg/gDW of tissue)

		FA	V	p.OHB	V / p.OHB
1	Vanilla Roots	257	165	539	0.31
2	Vanilla Roots + 0.5mM FA (3 days)	1995	541	529	1.02
3	Vanilla Roots + 0.5mM FA (3 days) + charcoal		33 (1101) 1068	673	1.63
4	As (3) (2 x 3 days) + charcoal		27 (3675) 3648	979	3.75
5	As 3 (5 x 3 days) + charcoal		40 (7100) 7060	910	7.80
6	Vanilla beans (dry)		15000	1170	12.81

CLAIMS

1. A method of producing natural vanillin, comprising reacting vanilla plant root material with ferulic acid or derivatives thereof in the presence of adsorbent material that releasably adsorbs vanillin; and extracting vanillin from the adsorbent material.

2. A method according to claim 1, wherein the adsorbent material is charcoal.

3. A method according to claim 1 or 2, wherein the adsorbent material is in finely divided or powdered form and is in direct contact with the root material.

4. A method according to claim 1, 2 or 3, wherein the adsorbent material is present at the start of the reaction.

5. A method according to anyone of the preceding claims, wherein the root material, ferulic acid (or derivatives) and adsorbent material are incubated together under suitable conditions for a period of about 3 days; the adsorbent material is then removed and treated to extract vanillin therefrom, while leaving unreacted ferulic acid (or derivatives) adsorbed thereto; and the root material is subjected to one or more further similar treatments with ferulic acid (or derivatives) for a further period of about 3 days with a new batch of adsorbent material followed by removal of the adsorbent material, and extraction of vanillin therefrom, with vanillin also being extracted from the root material after the final treatment.

6. A method according to claim 5, wherein the root material is subjected to a total of up to 6 treatments

each for a period of about 3 days.

7. A method according to any one of the preceding claims, wherein vanillin is extracted from the adsorbent material and the root material by mild alkaline hydrolysis followed by solvent extraction.

8. A method according to claim 7, wherein the solvent extraction is performed using diethyl ether.

9. A method according to any one of the preceding claims, wherein the adsorbent material is recycled for further use in the method of the invention after extraction of vanillin therefrom.

10. A method according to any one of the preceding claims, using ferulic acid.

11. A method according to claim 10, wherein the ferulic acid has been isolated from condensed molasses solubles (CMS) by treatment with charcoal.

12. A method according to claim 11, wherein the charcoal and extracted ferulic acid are used directly in the method of the invention with the charcoal constituting the adsorbent material.

13. A method according to claim 11 or 12, wherein the treated CMS is recycled for use as an animal feed supplement.

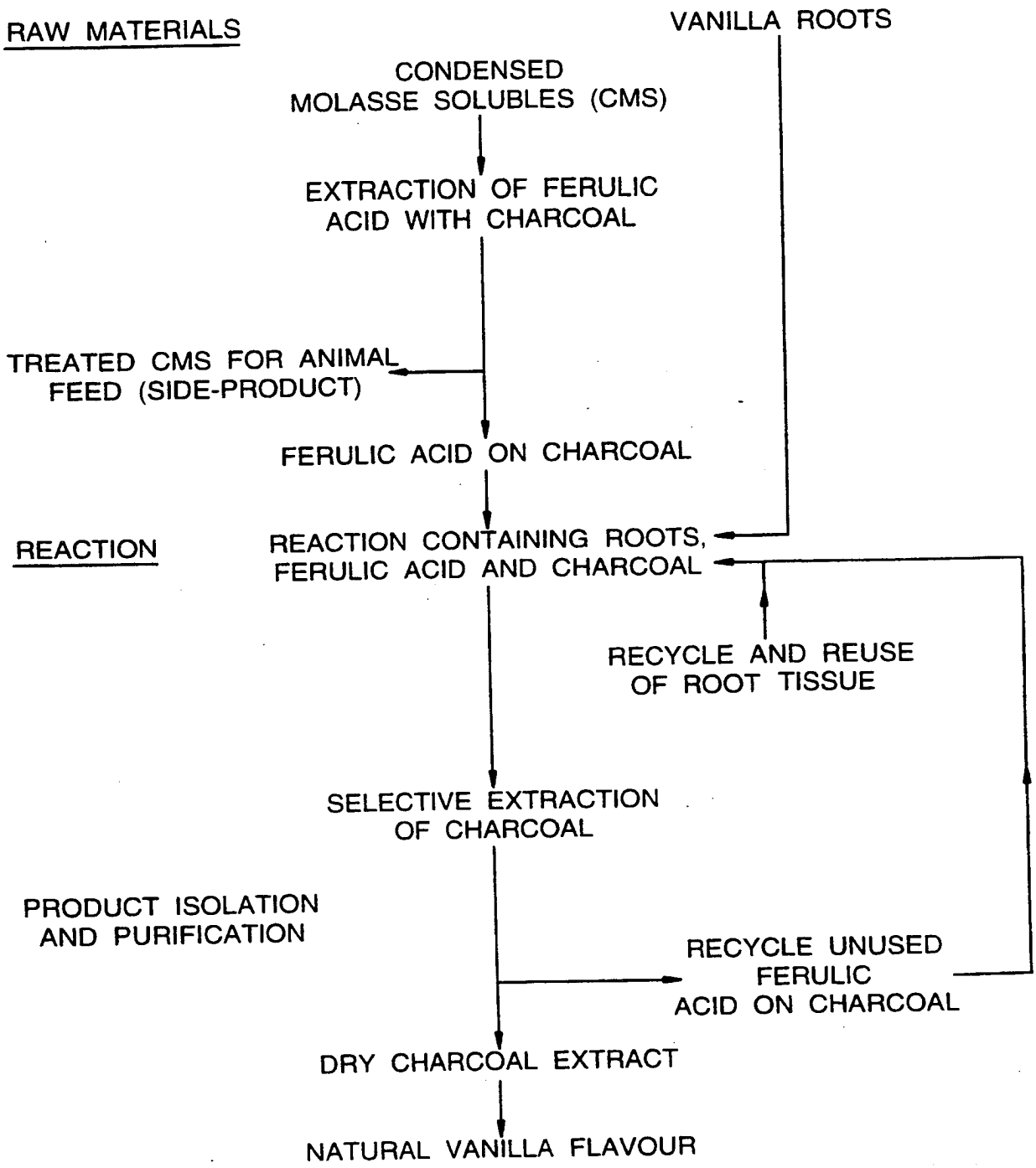
14. A method of producing natural vanillin, substantially as herein described.

15. Vanillin produced by the method of any one of claims 1 to 14.

16. A product comprising vanillin and p-hydroxybenzaldehyde at a vanillin: p-hydroxybenzaldehyde weight ratio about 7.8:1.

Fig.1.

PROPOSED PROCESS FOR PRODUCING VANILLA USING AERIAL ROOTS



INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	US,A,5 128 253 (I.M. LABUDA ET AL.) 7 July 1992 see the whole document -----	1

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